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Scaffolds for Spinal Cord Regeneration as a Treatment for Paralysis

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I. INTRODUCTION

The long-term objective of this research is to develop a device for treating spinal cord injury. The specific aims of the proposed study are to test new types of collagen tubes and porous collagen scaffolds. Moreover we will be investigating the effects of incorporating genes from nerve growth factors into the collagen scaffolds and seeding the scaffolds with marrow-derived mesenchymal stem cells. The standardized defect site is a 5-mm gap in the rat thoracic spinal cord. Our principal method of evaluation is histomorphometry.

Our supposition is that an appropriate synthetic substrate (*i.e.*, the collagen scaffold) will mimic or perhaps improve upon the documented ability of peripheral nerve grafts to promote the regrowth of injured spinal axons, and that the seeded stem cells will differentiate under the influence of the endogenous regulators to a support cell phenotype. Collagen-based materials are the bio-inspired biomaterials being used for the fabrication of the tube, covering film and porous scaffold.

The specific aims of this project are to investigate the effects of the following critical design variables on regeneration of the spinal cord in a rat model.

- a) Degradation rate of a collagen tube.
- b) Use of a collagen covering material to wrap around the ends of the stumps at the ends of a segmental defect in the spinal cord rather than insertion of the stumps into a tube.
- c) Use of a chondroitin sulfate containing porous collagen scaffold within the tube or wrap.
- d) Degradation rate of the porous collagen-GAG scaffold.
- e) Pore diameter of the porous collagen-GAG scaffold.
- f) Gene supplementation of the porous collagen-GAG scaffold.
- g) Seeding of the porous collagen-GAG scaffold with undifferentiated marrow-derived mesenchymal stem cells.
- h) Seeding of the porous collagen-GAG scaffold with differentiated marrow-derived mesenchymal stem cells.
- i) Seeding of the porous collagen-GAG scaffold with marrow-derived mesenchymal stem cells which have undergone gene transfer *ex vivo*.

II. BODY

During the past project year the following was accomplished, toward achieving the objectives of determining the effects of selected design variables on the reparative processes in spinal cord defects.

1. Development of methods to fabricate collagen tubes and porous cylindrical scaffolds from a porcine type I/III collagen material.
2. Implantation of collagen tube devices into the rat spinal cord model and processing of the tissue for histomorphometric evaluation.
3. Development of a method for the quantitative analysis of images of axons in the reparative tissue in the implant site.
4. Implementation of methods for the isolation and growth of marrow-derived mesenchymal stem cells.

A. Methods for the Fabrication of the Collagen Tubes and Porous Scaffolds

1. Collagen Tubes

Collagen tubes were fabricated from porcine type I collagen material containing a small amount of type III collagen. A high solids-content suspension of collagen (5% w/w) was

prepared by dissolving the collagen in a 0.05% acetic acid solution. The collagen suspension was degassed via centrifugation to remove air bubbles then injected into custom molds (Figs. 1-3) and lyophilized to form a collagen tube. The molds were fabricated from an aluminum shell with a Teflon insert that has semicircular channels milled down its length (Figs. 1 and 2). The two assembled halves of the mold formed a series of cylindrical channels bounded by Teflon (Fig. 2). The highly viscous collagen suspension was injected directly into each cylindrical channel and a mandrel, fabricated from a glass rod with a Teflon sleeve (Fig. 3), was inserted into the center of each channel to form the tubular geometry. Molds and mandrels with a range of diameters were machined in order to allow for the fabrication of collagen tubes with various internal and external diameters.

The collagen suspension in the molds was frozen in a freeze-dryer at a controlled temperature of -40°C. The frozen collagen suspension that formed around each mandrel was removed from the mold and the ice content of the solid removed by sublimation. After sublimation, the collagen tube was removed from the Teflon-coated mandrel (Fig. 4). The final tubes had an inner tube diameter necessary to accommodate the spinal cord stumps and a wall thickness of approximately 750 μm .

Two cross-linking processes, a physical and a chemical process, were employed to prepare implants with increasing cross-link density. Dehydrothermal cross-linking (DHT) was performed in a vacuum oven using temperature and time of treatment as variables to adjust the cross-link density to desired levels. The use of high vacuum (50mTorr) and temperature (90 - 120°C) results in severe dehydration of collagen, leading to the formation of covalent cross-links among the polypeptide chains of the collagen fibrils without converting the collagen into gelatin (*i.e.*, unraveling the collagen triple helical structure). The chemical cross-linking method was based on the use of a carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), to form zero-length cross-links between the collagen fibers. EDAC acts solely as a catalyst in the cross-linking reaction, allowing the cytotoxic carbodiimide to be completely rinsed from the matrix following the cross-linking reaction (22). The DHT process results in a much lower density of cross-links and faster expected degradation rates *in vivo* compared to the EDAC process.

Analyses of the tubes including a measure of permeability are in progress.

2. Porous Collagen-GAG Scaffolds

The porous collagen-GAG matrix was produced by freeze-drying a suspension of the type I/III porcine collagen. The collagen slurry was injected into holes in a polymeric plate 1.5 cm thick, which served as the mold. A copper plate was used as the base of the mold. As the copper plate was cooled ice crystallites formed in the slurry. After freezing the mold was placed in a freeze dryer. Pore analyses of the cylindrical porous rods are in progress.

B. Implantation of Collagen Tube Devices into the Rat Spinal Cord Model and Processing of the Tissue for Histomorphometric Evaluation

1. Implantation Procedure

The following animal study was approved by the Animal Care Committee of the VA Boston Healthcare System.

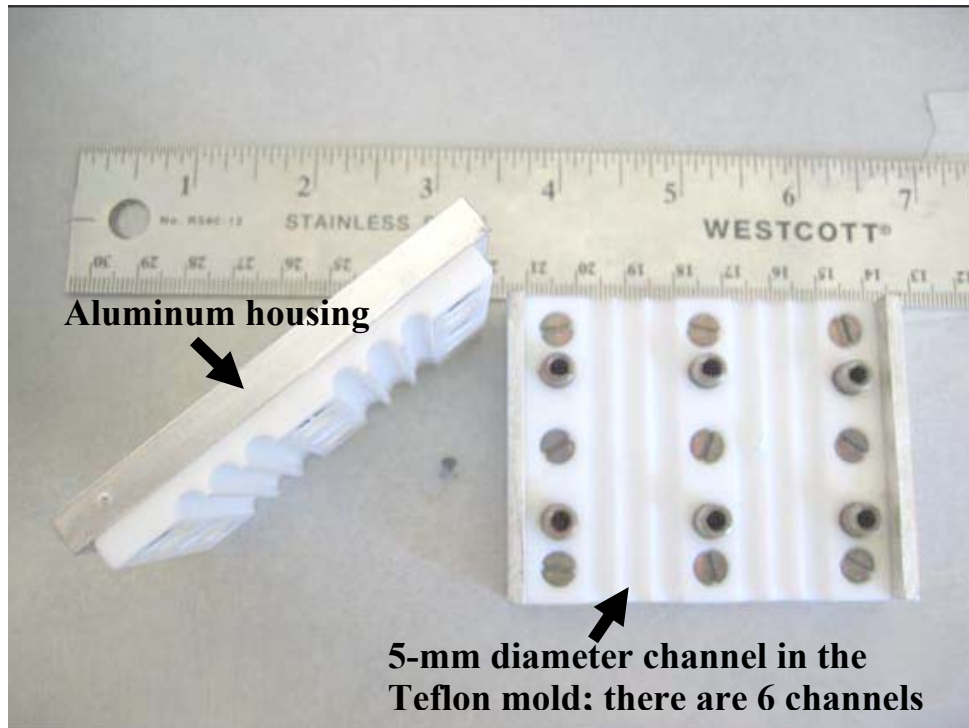


Fig. 1. Photograph of the mold used for the fabrication of the collagen tubes.

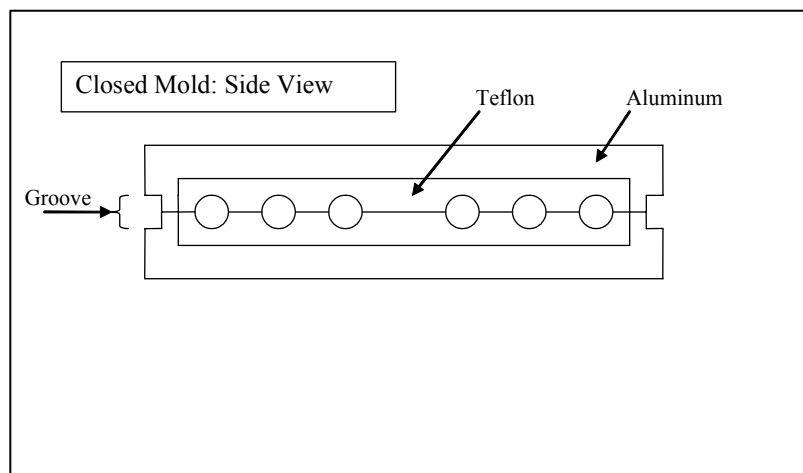


Fig. 2. Sketch of the mold used for the fabrication of the collagen tubes.

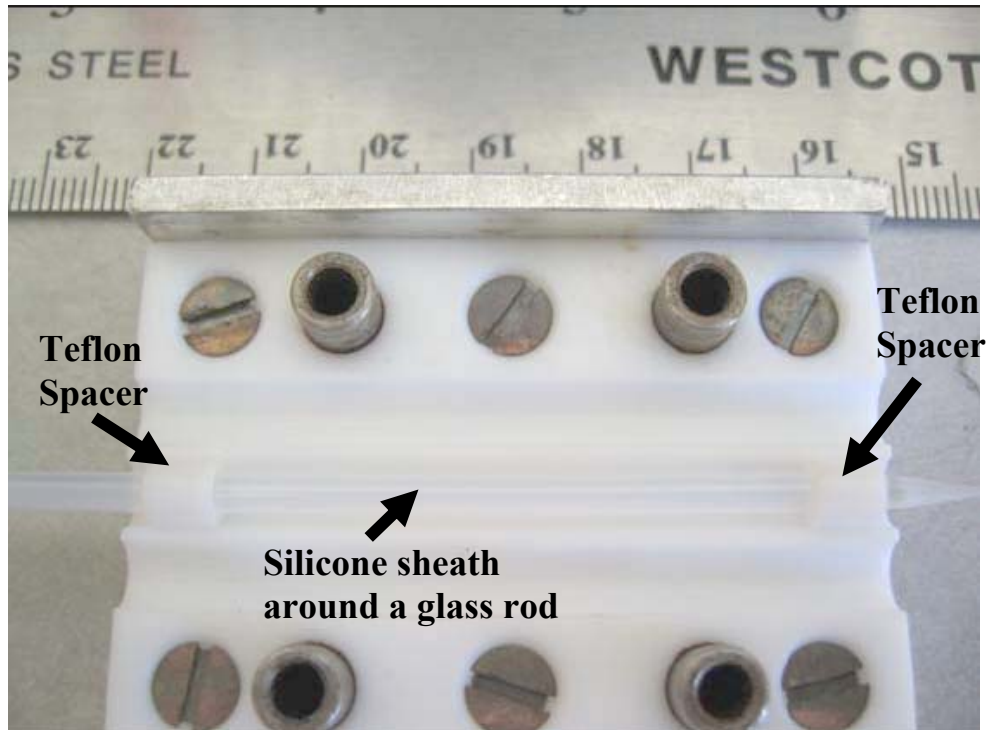


Fig. 3. Photograph of the mandrel in the channel of the mold used for the fabrication of the collagen tubes.



Fig. 4. Photograph of the tube molded from the slurry that was prepared by dissolving type I/III in acetic acid. The tube was dehydrothermally treated; it did not receive any additional cross-linking. The tubes handled well when dry and also after being hydrated. Of importance is that when the tubes were split longitudinally (with a blade) as shown in a later figure, the tubes would spring back to their original shape after being opened.

Table 1. Summary of the Numbers of Rats in Each Group*.
Groups are listed in the order in which the experiments were conducted.

IMPLANT GROUPS	Type of Rat¹	# Rats Operated On	# Died Intraop. and Prior to Scheduled Sac.	# Exhibiting Self-Mutilation	Final # of Rats in Group
1: Control; no implant	SD	14	6	4	8
2: Collagen dorsal barrier (covering membrane) alone	SD	10	2	4	8
3: Collagen membrane wrap w/o covering membrane	SD	10	6	3	4
4: Collagen tube split on top - w/o covering membrane	L	8	1	0	7
5: Collagen tube split on bottom + covering membrane	L	10	2	0	8
6: Control; no implant	L	4	1	0	3
7: Collagen tube split on top - + covering membrane	L	8	0	0	8
Totals	-	64	18	11	41

* Ten additional animals were sacrificed to yield marrow for the isolation of mesenchymal stem cells.

¹ SD, Sprague-Dawley; L, Lewis

Female, Sprague-Dawley rats weighing 250 to 300 grams were initially used in the study (Groups 1-3; Table 1). After it became clear that these animals displayed a high incidence of self-mutilation (Table 1; see section “3” below) Lewis rats were used (Groups 4-7; Table 1). Animals were anesthetized with sodium pentobarbital, and prior to surgery, antibiotics were injected subcutaneously to prevent post-operative infection. Hair was shaven from the back, and the skin cleaned with Butadiene. Surgery was performed under sterile conditions. A longitudinal incision, 5 cm in length, was made through the skin above the thoracic spine. The back musculature was incised along the midline and dissected away from the vertebral column. A dorsal laminectomy was performed between T7 and T10 using small bone rongeurs and microscissors. The dura was opened with a surgical blade to expose about 10 mm of spinal cord. Two spinal cord segments (T8 and T9) were removed by performing two complete transections and removing the intervening tissue. This resulted in a cord gap, 5 mm in length. Complete spinal cord transection and removal of a segment of tissue ensured unambiguously that all axonal pathways had been disrupted. The nearest pair of spinal roots entering the intact rostral (T7) and caudal (T10) spinal cord was severed.

After implantation of the collagen device, a collagen membrane was placed over the implant site as a dorsal barrier to prevent the collapse of overlying tissues into the implant site. The wound was then closed.

Following surgery, rats were placed on a heating pad to maintain body temperature until regaining consciousness in 2-4 hours. Post-operatively animals lacked normal micturition reflex, and their bladders had to be emptied manually by the method of Credé twice daily until spontaneous voiding occurred.

2. Experimental Groups

One of the initial objectives of the experimentation was to determine the effects of entubulating the gap in the spinal cord in a collagen tube. During the course of such implantation it became clear that the stumps of the spinal cord were being damaged by the manipulations required to insert them into the tube. Therefore, alternative approaches were tested:

- a) wrapping a collagen membrane around the stumps to thereby entubulate the gap (Group 3 in Table 1);
- b) splitting the collagen tube to open it to more easily entubulate the gap with the split placed at the top (Groups 4 and 7 in Table 1 and Fig. 5); and
- c) cutting away a portion of the tube so that it could fit over the gap site with minimal disturbance to the cut ends of the cord (Group 5 in Table 1; Fig. 6).

Groups 1 and 2 (Table 1) were control groups.

Tissue samples from the animals in the groups in Table 1 are currently being evaluated.

3. Self-Mutilation

We found that 11 of the first 34 Sprague-Dawley rats employed in the study displayed self-mutilation (Table 1). The severity of the wounds varied greatly but generally required humane sacrifice of the animals before the scheduled period. We subsequently switched to Lewis rats and found no such problem with self-mutilation (Table 1).

4. Histological Processing of Tissues

At sacrifice the skin and muscles above the spine were opened, and the spinal cord wound site located. The entire thoracic spinal cord including the lesion was removed and further fixed in 4% paraformaldehyde for 4 hours at 4°C. Tissue from the midpoint of each wound was fixed overnight in mixed aldehydes, postfixed in buffered 1% osmium tetroxide for 2 hours, dehydrated through graded alcohol, and embedded in Epon. Transverse sections for light microscopy were cut at 1.5 μm thickness and stained with toluidine blue. Digitized images captured at 4X objective magnification were used to determine the cross-sectional area (in mm^2) of tissue at the midpoint of the gap, and images captured at 100X were used to count the number of myelinated axons and determine their aspect ratio.

The ratio of the largest diameter to the smallest diameter (aspect ratio) for each myelinated fiber is being calculated as a measure of the degree to which regrowing axons have followed a direct path between spinal cord stumps. Axons that had elongated nearly parallel to the long axis of the spinal cord appear nearly circular in transverse histological sections (aspect ratio near 1.0) while axons that were traveling obliquely to the spinal cord axis appear as ellipses in cross section (aspect ratio greater than 1.0).

Tissue comprising the transition between rostral and caudal spinal cord and tissue in the gap was removed from immersion in 4% paraformaldehyde and placed in a solution of 30% sucrose in PBS for an additional 18-24 hours. Tissue samples were frozen by immersion in isopentane at -40°C and stored at -80°C. Longitudinal sections are being cut on a cryostat at 15 μm thickness

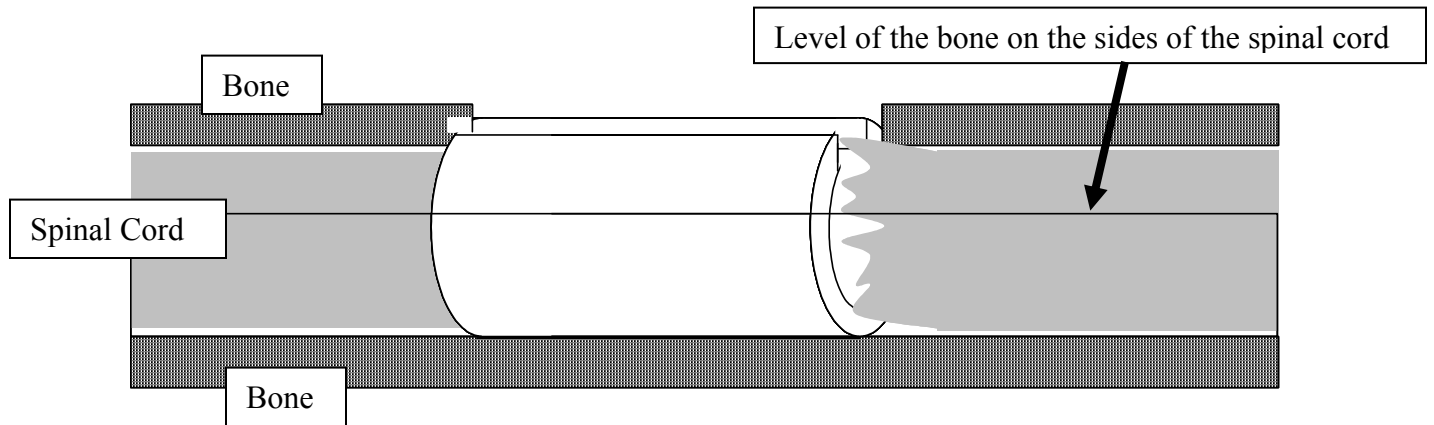


Fig. 5. This sketch shows how the tube was implanted in Groups 4 and 7 (Table 1). It was much easier to insert the ends of the spinal cord into the tube after the tube was split and opened. After the stumps of the cord were inserted the tube, the tube automatically recovered its original shape. The split ends of the tube came together that it was not necessary to suture the tube closed.

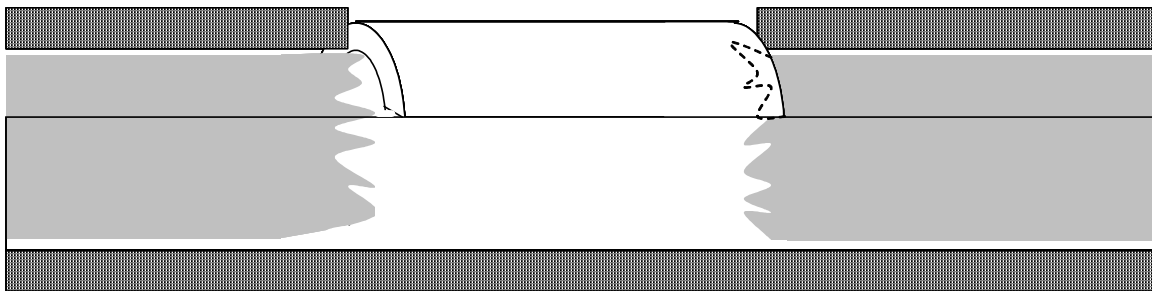


Fig. 6. This sketch shows how the tube will be implanted in Group 5 (Table 1). A portion of the tube was cut away before it was implanted. It rested on to of the spinal cord stumps and covered the gap.

and stained with hematoxylin and eosin (H&E) for general cell identification or Masson's trichrome to identify collagen. Adjacent sections are being captured on slides for immunohistochemistry. Astrocytes will be identified using polyclonal rabbit anti-glial fibrillary acidic protein antibody (anti-GFAP; Incstar, Stillwater, MN) diluted 1:6. Axons will be identified using mouse monoclonal anti-68 kDa neurofilament antibody (Oncogene Sciences, Uniondale, NJ) diluted 1:400. Additional tissue sections will be similarly processed except for the replacement of each primary antibody with non-immune serum as controls to verify the antibody specificity.

C. Development of a Method for the Quantitative Analysis of Images of Axons in the Reparative Tissue in the Implant Site

The cross-section of the center of the lesion (spinal cord transection) in the thoracic section of the adult rat spinal cord was evaluated for the presence of regenerated myelinated axons by staining explanted spinal cord specimens with osmium tetroxide (which stains for myelin). The specimens were embedded in plastic for sectioning and then stained with toluidine blue to provide greater contrast among cellular elements.

Cross-sectional images of the lesion center were then captured using light microscopy and a digital camera. Images were captured first at low magnification (40x) in order to view the entire lesion cross-section (Fig. 7) and then additional images were captured at 200x magnification to provide sufficient resolution for the identification of individual axons (Fig. 8).

The images were analyzed using ImageJ software (available as freeware from NIH, Bethesda, MD). This software can be utilized to measure various parameters in the histological sections including: the total number of axons, the area of each individual axon, the average axonal area and standard deviation, the percentage of the cross-sectional area of the spinal cord covered by axons, and the major and minor axes of elliptical approximations of each axon.

The process for analyzing the sections involved converting the image to black and white and increasing the contrast until the axons were clearly outlined (Fig. 9). This allowed the ImageJ software to be able to distinguish the axons and quantify them. Figure 10 shows the outlines and numbering produced by the use of the ImageJ software. The software outputs data for each labeled axon such as area, major and minor axes, total number of axons and other information.

The image showing the outlines of the detected axons (Fig. 10) can then be compared to the original unaltered micrograph (Fig. 8) by overlaying the images to validate the accuracy of the software. Axons which are not picked up by the software can then be manually outlined to ensure they are identified in subsequent image analysis.

This method is currently being used to analyze the samples from the groups in Table 1.

D. Isolation and Growth of Marrow-Derived Mesenchymal Stem Cells

1. Rat Mesenchymal Stem Cell Harvesting and Culture

Rat mesenchymal stem cells (MSCs) were harvested from the bone marrow of the femurs and tibias of adult female Lewis rats. Under sterile conditions, the tibias and femurs were removed from the animals and the bones were thoroughly cleaned with a knife to remove attached muscle, periosteum, cartilage and ligaments. The bones were then crushed using a mortar and pestle in the presence of culture medium. The culture medium consisted of low-glucose Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), and 1% antibiotics. The crushed bones were then rinsed with additional culture medium and filtered

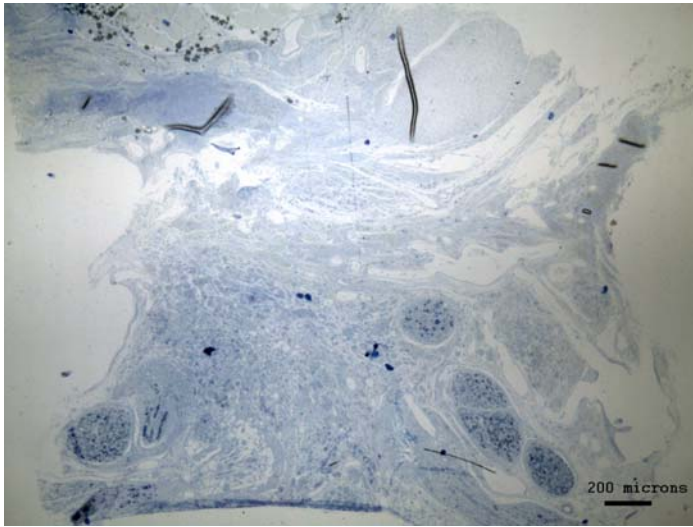


Fig. 7. Cross-section of the center of the lesion of a control animal (no implantation). (40x)

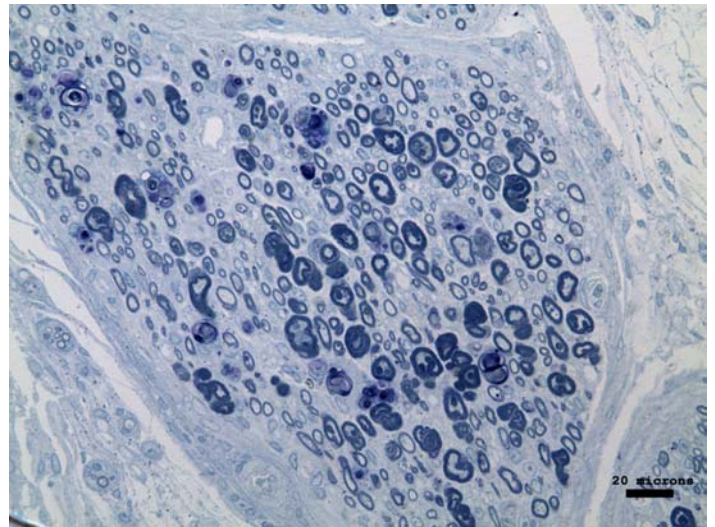


Fig. 8. 200x magnification of the lesion center of a control animal.

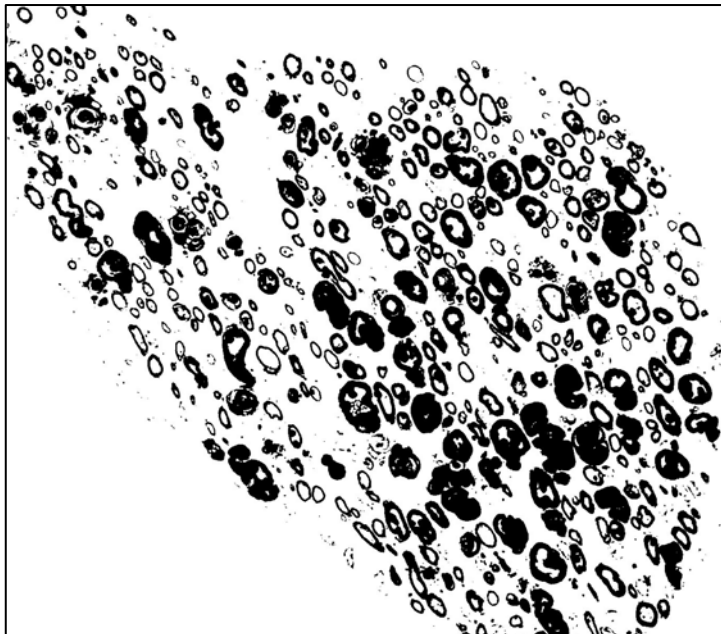


Fig. 9. High contrast black and white image of spinal cord section.

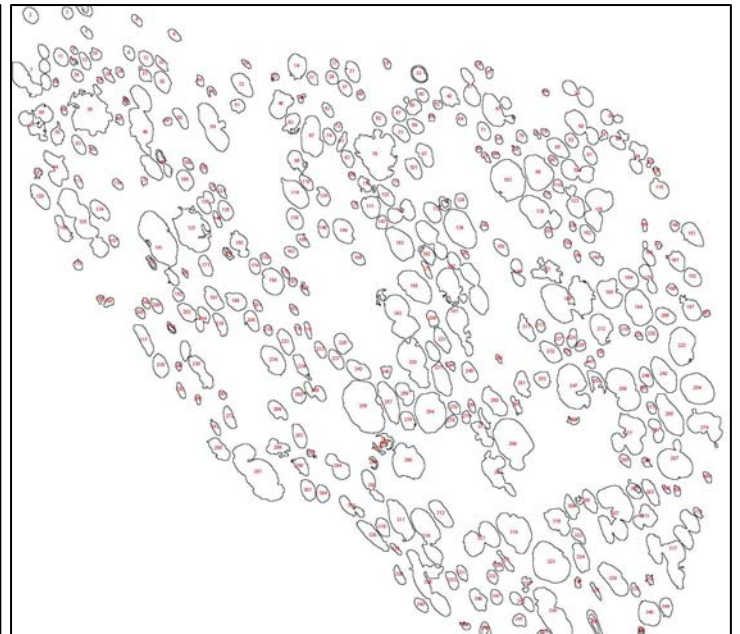


Fig. 10. Numerical labeling and outlining of individual axons.

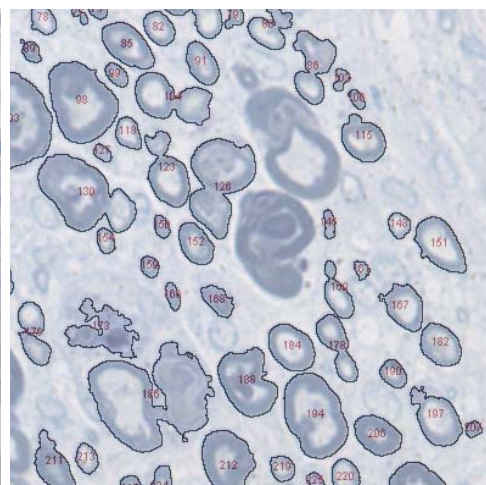
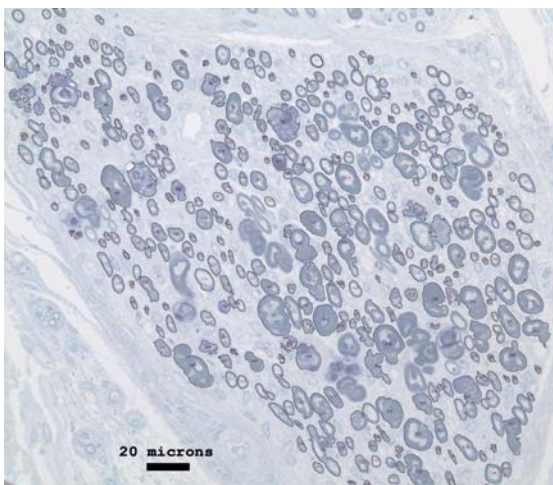


Fig. 11. Original image (left) overlaid with outlines of identified axons using ImageJ (rt.).

twice using 40µm cell filters. The cells were centrifuged, rinsed with phosphate buffered saline (PBS) and resuspended in culture medium. Cells from one rat were plated in one 75cm² cell culture flask and cultured at 37°C with 5% CO₂. After 48 hours the non-adherent cells were removed (by medium change) and the attached cells were cultured until they grew to approximately 90% confluence. The culture medium was change every other day.

Cells were passaged once they reach 90% confluence. The primary culture cells were washed with PBS and detached by incubation with Trypsin and EDTA. Detached cells were counted and frozen for future use in aliquots of 0.5 to 0.7 ml at a cell concentration of 1 million cells per ml.

2. Chondrogenic Assay Protocol

In order to demonstrate the chondrogenic differentiation potential of the rat MSCs, as a means of characterizing the cells as MSCs, a 3-dimensional bioassay was performed to induce cartilage production. Primary culture MSCs were grown to confluence and passaged. A high concentration cell suspension was produced in a small volume of PBS. An appropriate volume of the cell suspension was added to 15ml centrifuge tubes to achieve 200,000 cells per tube. Chondrogenic medium (0.5ml; see below) was added to each tube. The cells were centrifuged for 10 minutes at 1500 RPM and the tubes were placed in an incubator. Small pellets appeared within 2 to 3 days. The medium was changed every other day and the pellets were fixed for histological evaluation after 2 weeks.

The chondrogenic medium consisted of high-glucose DMEM, 1% MEM nonessential amino acids, 1% Hepes buffer, 1% penicillin, streptomycin and glutamate (PSG), 1% ITS+1, 7.5 g/ml of BSA solution, 10ng/ml of TGFβ-1, 100nM of dexamethasone, and 0.1mM of L-ascorbic acid 2-phosphate.

III. KEY RESEARCH ACCOMPLISHMENTS

- Development of methods to fabricate collagen tubes and porous cylindrical scaffolds from a porcine type I/III collagen material.
- Implantation of collagen tube devices into the rat spinal cord model.
- Development of a method for the quantitative analysis of images of axons in the reparative tissue in spinal cord gaps.

IV. REPORTABLE OUTCOMES

1. Development of methods to fabricate collagen tubes and porous cylindrical scaffolds from a porcine type I/III collagen material.
2. Rat spinal cord model providing a standardized defect in which to test devices for spinal cord regeneration.
3. A method for the quantitative analysis of images of axons in the reparative tissue in a defect in the spinal cord.

V. CONCLUSIONS

Absorbable collagen tubes, suitable for implantation in surgically created gaps in the rat spinal cord, can be fabricated from type I/III porcine collagen. The tubes can be implanted into such defects in a configuration that causes a minimal amount of additional trauma to the cut ends of the spinal cord. The number and diameter distribution of axons that have re-grown across the

gap in the collagen tube can be quantified using a novel image analysis method in conjunction with light microscopy.

VI. REFERENCES

None

VII. APPENDICES

None